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(54) Method of ovulation detection and indicator strip for use therein

(57) A method is provided for determining the concentration of pregnanediol glucuronide (PG) in a woman's urine which is characterized by utilization of the reagent 20 α -hydroxy-4-pregnen-3-one (20 α -reagent) in a form in which it reacts with antibodies binding to PG. The method is adaptable to a visual color indication test which can be performed by the woman herself as well as by laboratory analysis. The method can be used to define the period in which conception can occur, to define a post-ovulation safe period in which conception is prevented, and as an early pregnancy indicator.

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SPECIFICATION

Method of ovulation detection and indicator strip for use therein

- 5 The field of this invention relates to measurement of the concentration of the hormone pregnanediol in women's urine. In urine, this hormone occurs in the form of pregnanediol glucuronide (PG) and is detected in that form. The amount of PG in a woman's urine rises rapidly at the time of ovulation, and declines rapidly at the time of menstruation. However, if conception has occurred, the PG level remains high. Therefore, measurement of pregnanediol 10 levels in urine is a potential usable procedure for either preventing or diagnosing pregnancy.
- 10 Although ovulation may be detected by characteristic in basal body temperature or in physical properties of cervical mucus, these methods are subjected to variability from body functions unrelated to ovarian function and therefore not sufficiently reliable for detecting ovulation in many women.
- 15 Although pregnanediol is the major urinary product of progesterone, pregnanediol assays have not been widely utilized for assessment of luteal function. There are basically two reasons for this. First, earlier methods have required the collection of 24-hour urine specimens, an unreasonable request for even highly motivated patients when, to be most useful, collections should be made daily for at least 10 days during the middle of the menstrual cycle. Second, the assay procedure has been laborious, requiring usually two days for completion, and is subject to errors from several sources; the necessity for enzymatic or acid hydrolysis of the glucuronide moiety for colorimetry or gas chromatography is the most difficult and variable aspect of the assay.
- 20 It has been demonstrated that measurements of PG levels in overnight urine specimens correspond closely with 24-hour specimens. See Judge, et al, *Steroids* 31: 175-187 (1978).
- 25 The reported tests were conducted on a laboratory basis by a gas chromatographic method. Samarajeewa, et al have published a method for preparing antiserum specific for PG. J. *Steroid Biochem.* 11: 1165-1171 (1979). Pregnanediol in its free acid form was joined covalently to bovine serum albumin, and the antisera was prepared in rabbits. A calibration curve was prepared with radio-labelled PG, as part of the development of direct radioimmunoassay procedure for PG in women's urine. Specificity was established by showing that other steroids present in urine did not cross-react.
- 30 The antiserum prepared as described by Samarajeewa, et al; was used by Collins et al in radioimmunoassay procedure for measuring PG in overnight and 24-hour urine samples. *Acta Endocrinologica* 90: 336-348 (1979). The results confirmed the correlation previously observed by Judge et al. In the reported assay procedure, radio-labelled PG was employed as the test reagent for competing with the urine PG in binding to the antibodies of the immune serum. Thus, an expensive and relatively unstable reagent, pregnanediol glucuronide was required both for preparing the antiserum and as a radio-labelled test reagent, and the radioimmunoassay was carried out on a laboratory basis requiring special equipment. Heretofore, no one has provided the ovulation detection art with a simple, inexpensive and accurate procedure for determining 35 levels of PG in urine which can be employed by women on a home-use basis.
- 35 This invention is based in part on the discovery that a commercially available relatively inexpensive steroid can be substituted for pregnanediol glucuronide in assay procedures using PG-antiserum. This steroid is 20 α -hydroxy-4-pregn-3-one (referred to herein as the 20 α reagent). Further, if desired, the antibodies binding to PG can be produced by using the 20 α reagent as the antigen. Even more importantly, however, the 20 α reagent can be used as the basis of a test kit for home-use by women to determine PG in overnight urine samples, the level of PG in the urine being indicated by a visual color comparison. In one preferred embodiment, 40 the 20 α reagent is immobilized on a solid adsorbent in the form of a fibrous strip, such as glass fiber paper, which can be dipped into the urine for reaction with the antibodies therein which have not bound to the PG of the urine. The amount of antibodies adsorbed on the strip can be indicated by having the antibodies labelled with a substance capable of providing a color reaction, such as a color compound like rhodamine, or an enzyme such as peroxidase which will react to produce a color. Alternatively, the amount of antibody protein bound to the test strip 45 can be indicated by applying a protein staining dye such as Coomassie blue. Women are therefore provided with a simple, inexpensive and effective method for defining the time of ovulation. The woman may then use this information either to promote or prevent conception, or as a method for early self-diagnosis of pregnancy.
- 50 The method and indicator strip of the present invention utilize a 20 α reagent which comprises 20 α -hydroxy-4-pregn-3-one as the base reagent. The 20 α reagent as used in this invention is preferably in the form of a conjugated derivative which reacts readily with antibodies binding to pregnanediol glucuronide (PG). For example, the 20 α reagent can be conjugated through the C-3 of the steroid to a solid support material or to an antigenic protein.
- 55 For example, a suitable coupling group is introduced at the C-3 position of the 20 α reagent.

This does not modify the antigenic properties of the reagent, while adapting it for attachment to a glass fiber strip or other solid support, or to a suitable antigenic protein.

The other required reagent is the antiserum containing the antibodies binding to PG. This can be prepared by hyperimmunizing animals, such as rabbits, to a conjugate of PG and an

- 5 antigenic protein, such as bovine thyroglobulin. For example, the procedure described by Samarajeewa, et al, *J. Steroid Biochem.* 11: 1165-1171 (1979), can be used. The 20- α reagent is conjugated to a suitable antigenic protein and used to prepare the antiserum. The antiserum obtained provides anti-PG antibodies that bind to the 20- α reagent either as the free steroid or as its C-3 derivatives.
- 10 In general, the method of this invention for determining the concentration of PG in a woman's urine is characterized by the steps of reacting a predetermined amount of anti-PG antibodies with a measured amount of the urine to leave a definite amount of unreacted antibodies for reaction with the 20- α reagent on the strip, or the antibodies are reacted with a measured amount of the urine which contains a quantity of a colored derivative of the 20- α reagent so that
- 15 15 the reagent and the PG in the urine competitively react with anti-PG antibodies on the strip. In either case, after the reaction, either the amount of the reacted antibodies or the amount of unreacted 20- α reagent is determined. For example, this can be done by radio-labelling of the antibodies or the 20- α reagent. The radioimmunoassay may be conducted, in general, as described by Collins, et al. *Acta Endocrinologica* 90: 336-348 (1979), except that the radio-labelled 20- α reagent would be employed instead of radio-labelled PG. However, to achieve the 20 full benefits of the present invention, it is preferred to employ a visual immunoassay procedure employing a color indicator.

In practicing the present invention, either a predetermined quantity of the antibodies or a predetermined quantity of the 20- α reagent can be immobilized on a color indicator support. For 25 example, the support may comprise an adsorbent fibrous strip, such as that composed of porous glass beads or glass fiber paper. The antibodies may be attached to a solid surface without interfering with their binding capacity using the method described by Robbins and Schneerson, *Methods in Enzymology* 34: 703-731 (1974). Similarly, 20- α reagent may be bound to the glass without interfering with its reactivity with the antibodies by the method of Parikh et al, 30 *Methods in Enzymology* 34: 670-688 (1974). General methods of conjugation to glass surfaces are described by Weetall and Filbert, *Methods in Enzymology* 34: 59-71 (1974).

In one preferred embodiment, a precise quantity of the 20- α reagent, provided with a coupling group at its C-3, is bound to a small test strip, such as a piece of glass fiber filter paper, and means are provided for dipping the entire strip in the urine. Such means may 35 comprise tweezers, or the test strip may be attached to the end of a rod, the assembly providing a dipstick. Preferably, the antibodies to be added to the urine are labelled with a substance capable of providing a visual color indication. For example, the antibodies may be conjugated to a dye such as rhodamine. A suitable procedure for such conjugation is described in *J. Immunol. Methods*, 13: 305 (1976). After the reaction of the antibodies with the PG in the urine sample, 40 the remaining unreacted antibodies are adsorbed by the 20- α reagent on the test strip. The intensity of the color will depend on the amount of the antibodies adsorbed. Further, additionally or alternatively, the antibodies after adsorption can be stained with a protein-staining dye such as Coomassie blue, bromophenol blue or tetrabromophenolphthalein ethyl ester.

One especially desirable procedure providing a high degree of color sensitivity is to conjugate 45 the antibody to an enzyme which will produce a color reaction in a specific developing solution. For example, in one preferred procedure, the antibodies are conjugated to the enzyme peroxidase. This can be done by the method of Avrameas, *Methods in Enzymology* 44: 709-717 (1976). The unreacted portion of the antibodies are then adsorbed from the urine sample by the 20- α reagent on the test strip. The color is then developed by immersing the test 50 strip in 0.05% of 3,3'-diaminobenzidine in pH 7.6 buffer. Hydrogen peroxide (2 drops of 3% solution) is added for color development. This procedure is also described in the article by Avrameas cited above.

In other procedures which could be employed, the 20- α reagent is conjugated to a color indicator dye such as rhodamine, and is used in the urine sample. A suitable procedure for such 55 conjugation is described in *J. Steroid Biochem.*, 13: 489-493 (1979). However, the sample of urine containing the color-indicator conjugated 20- α reagent is then contacted with the test strip for competitive adsorption of the PG and 20- α reagent. This procedure can provide a color indication of the amount of PG in the urine, it does not give as great a sensitivity or as clear a color discrimination as the procedures described above.

60 The technical basis for this invention and various embodiments thereof are further illustrated by the following examples.

EXAMPLE I

Antisera was produced in rabbits that were immunized by 3 intradermal injections 3 weeks apart with pregnanediol glucuronide that had been conjugated to bovine thyroglobulin via the 65

mixed anhydride reaction. See Erlanger, et al, The Preparation of Steroid-Protein Conjugates to Elicit Antihormonal Antibodies, in Williams and Chase, *Methods in Immunology and Immunochemistry*, Vol. 1: Preparation of Antigens and Antibodies, New York, Academic Press, 1967; and, Kellie, et al, Steroid Glucuronide-BSA Complexes as Antigens, The Radioimmunoassay of 5 Steroid Conjugates, *J. Steroid Biochem.* 3: 275-288 (1972). The conjugate had been purified by dialysis and by precipitation from acetone. A 5 mg sample of the product was analyzed after it was hydrolyzed by heating in 0.75 N HCl in 66% (v/v) acetic acid at 100°C. for 15 minutes. The liberated steroid was extracted from the cooled, neutralized reaction mixture with ethyl acetate and was analyzed by gas chromatography. A molar ratio of steroid to protein of 157/1 10 was found. For each injection the conjugate was suspended in 1.0 ml of 0.9% NaCl and emulsified with an equal volume of Freund's incomplete adjuvant (the first injection in each animal was made with Freund's complete adjuvant). Rabbits received 2.5 mg of conjugate on each of the first 3 injections. Booster injections, also intradermal, were 0.25 mg. The titer of antisera was assessed by determination of the percent binding of ^3H -pregnanediol in doubling 15 dilutions of sera. The antiserum (0.2 ml) bound 50% of 15,000 dpm of ^3H -pregnanediol at a serum dilution of approximately 1/5000.

EXAMPLE II

In order to test the specificity of pregnanediol glucuronide antiserum prepared as described in 20 Example I, competition of several unlabelled steroids for ^3H -pregnanediol was measured. Table A summarizes the activity of these steroids in terms of the inverse of the concentration of competitor divided by the inverse of the concentration of pregnanediol that is required for 50% decreases in binding of the labelled ligand. The antiserum was found to be highly specific for the 20- α reagent of this invention as well as for pregnanediol and its glucuronide, the reactivity 25 of the 20- α reagent being of the same order as shown by the tabulated results.

Table A
Specificity of Anti-pregnaneadiol Glucuronide Antiserum

30	Steroid	% Competition	30
5 β -Pregnane-3 α ,20 α -diol (pregnanediol)	100		
Pregnaneadiol glucuronide	133		35
35 20 α -hydroxy-4-pregnen-3-one (20- α reagent)	126		
5 β -Pregnane-3 α ,20 α -diol	0		
5 β -Pregnane-3 α ,17 α ,20 α -triol	0		
5 β -Pregnane-3 α ,20 α -diol diacetate	0		40
40 Progesterone	0		
Esteradiol	0		
Esteriol	0		
Androsterone	0		
Cortisol	0		45
45 Corticosterone	0		

EXAMPLE III

The 20- α reagent can be bound to glass fiber paper after reacting the cleaned paper with 50 10% (v/v) 3-aminopropyltriethoxysilane in toluene. The free amino groups in this product can be cross-linked in 2.5% glutaraldehyde to polylysine (mol. wt. about 50,000). This provides a suitable support for covalently binding C-3 coupling derivatives of 20- α . For this purpose the 3-(O-carboxymethyl) oxime can be prepared in high yield by treatment of the 20- α steroid with aminoxyacetate in pyridine. This product can be conjugated directly, via the carbodiimide 55 product promoted condensation, with the solid support (see Erlanger et al, cited in Example I), or a "bridge" such as p-aminobenzoic acid can be inserted between the steroid oxime and polylysine to decrease steric hindrance in the binding of antibody to the 20- α . See Weetall and Filbert, *Methods In Enzymology*, 34: 59-71 (1974). Using the carbodiimide procedure, 0.02 μ moles of steroid can be conjugated to 1 cm² of the solid support. This is more than 20 times 60 the amount necessary to bind all of the antipregnaneadiol antibody (ADP) that is required for the visual assay. An excess of binding capacity is needed to separate the ADP that has not already bound pregnaneadiol glucuronide from the solution.

The glass fiber filter paper (or other solid surface) with the 20- α attached can be fastened to the inside surface of a tube in which the competition reaction takes place, or to a "dipstick" 65 with an epoxy cement.

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EXAMPLE IV

A preferred form the visual assay system employs 20- α bound to a solid support within a test tube. Sufficient lyophilized APD (purified IgG fraction) is added to the test tube to bind the 5 amount of pregnanediol glucuronide typically found in 0.1 ml of urine from women six days after ovulation (0.0007 μ moles with an antibody valence of 2).

The person using the assay adds 0.1 ml of urine and an ampule containing 0.9ml of buffer to the test tube that is already prepared as described above, and mixes the solution in the tube briefly. After 15 minutes, the solution is poured out, rinsed with water, and the indicator dye 10 (Coomassie blue) is added. The color that develops within a few minutes is stable for several days, and can be compared with a color chart to determine the approximate level of pregnanediol glucuronide in urine.

The composition of the dye is that described by Bradford (*Anal. Biochem.* 72: 248, 1976). The darkest blue color develops when no pregnanediol glucuronide is present in the urine 15 specimen, i.e., all of the APD is bound by the 20- α on the solid support. When the highest concentrations of pregnanediol glucuronide occur, e.g., after conception, the color will be light brown since very little APD will be available to bind to 20- α on the solid support and will be rinsed out of the tube. Intermediate concentrations of pregnanediol glucuronide in urine will give intermediate intensities of blue in the dye solution.

EXAMPLE V

In a procedure providing even greater reading sensitivity than that of Example IV, the antibodies are conjugated to peroxidase (Avrameas, *Methods In Enzymology*, 44: 709-717, 1976), and added to the urine in that form. The amount of antibody picked up and bound to a 20- α on the solid support in this case is measured by the color that develops on the surface of the solid support when the peroxidase enzyme that is conjugated to APD reacts with a substrate for a definite period of time. One preferred procedure is to add 0.5 mg of 3,3'-diaminobenzidine tetrahydrochloride in 1.0 ml of 0.1 M Tris-HCl buffer, pH 7.6. After adding a drop of 3% H₂O₂, the mixture is incubated for 5 min. at room temperature. The solution is poured out and the 25 tube rinsed with water. Staining of the antibody on the solid support surface is proportional to the amount of antibody present. Quantification is done by the use of a color chart as with the procedure employing Coomassie blue.

EXAMPLE VI

35 The procedure for using the 20- α reagent in a radioimmunoassay requires that the tritiated form, [1,2-³H(N)] 20 α -hydroxy-4-pregn-3-one, which is available from New England Nuclear Corp., 549 Albany Street, Boston, MA, be substituted for the compound (pregnanediol glucuronide) to be measured. Antiserum (APD) prepared as described earlier is diluted until a concentration that binds approximately 70% of 20,000 dpm of tritiated 20- α (specific activity 40 approximately 50 Ci/mmol) is obtained. This dilution of APD with the [³H] 20- α is used to set up a standard curve in which either 20- α or pregnanediol glucuronide can be used as the steroid that is the competitor for [³H] 20- α . If unlabelled 20- α is used as the competitor, a mathematical correction may have to be made for the difference in binding affinity of 20- α and pregnanediol glucuronide. Otherwise the procedures for radioimmunoassay are the standard methods.

45 Urine to be assayed is diluted 1/50, 1/100, and 1/500 with Buffer A (0.1 M sodium phosphate, 0.15 M sodium chloride, 0.015 M sodium azide, pH 7.0, containing 0.1% gelatin), and 0.1 ml aliquots of each dilution are added to assay tubes. Then 0.1 ml of the diluted antiserum (APD) containing 20,000 dpm of [³H] 20- α is added to each sample. After incubation of the samples and standards for 2 hrs at room temperature, the tubes are placed in a 4°C cold 50 room for 1/2 hour. Then, 0.2 ml of a suspension of dextran-coated charcoal (250 mg activated charcoal and 25 mg of dextran in 100 ml of Buffer A) is added to each tube. The samples are centrifuged to sediment the charcoal, and aliquots of the [³H] 20- α bound to the diluted APD in solution are counted in a liquid scintillation counter.

The counting method and calculation of results by comparison of displacement of [³H] 20- α 55 from APD by samples and standards follow usual procedures.

CLAIMS

1. A method of determining the concentration of pregnanediol glucuronide (PG) in a woman's urine, comprising the steps of reacting antibodies binding to PG with a predetermined quantity of a 20- α reagent consisting essentially of 20 α -hydroxy-4-pregn-3-one, either a predetermined quantity of said antibodies having been previously reacted with a measured amount of the woman's urine leaving a corresponding amount of unreacted antibodies for reaction with said 20- α reagent, or said quantity of antibodies being reacted with a measured amount of the woman's urine which contains said quantity of said 20- α reagent so that said 60 reagent and the PG in said urine competitively react with said antibodies, and thereafter 65 measured.

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determining either the amount of unreacted antibodies or the amount of unreacted 20- α reagent.

2. A method according to Claim 1 in which said predetermined quantity of antibodies is immobilized on a color indicator support, and said 20- α reagent in said urine is labelled with a substance capable of providing a visual color indication of the amount of reacted antibodies. 5

3. A method according to Claim 1 in which said predetermined quantity of 20- α reagent is immobilized on a color indicator support and said predetermined quantity of said antibodies is in the urine, in which after said reaction a protein indicator dye is applied to said support to provide a color indication of the amount of unreacted antibodies thereon. 10

10 4. A method according to Claim 2 in which said 20- α reagent is conjugated to a color indicator dye. 10

5. A method according to Claim 4 in which said dye is rhodamine.

6. A method according to Claim 1 in which said predetermined quantity of said 20- α reagent is immobilized on a color indicator support and said predetermined quantity of said 15 antibodies is in said urine, and said antibodies are conjugated to an enzyme capable of providing a color indication reaction. 15

7. A method according to Claim 6 in which said enzyme is peroxidase.

8. A method according to Claim 3 of Claim 4 in which said support is an adsorbent fibrous strip which can be dipped into said urine. 20

20 9. A method according to Claim 6 or Claim 7 in which said support is an adsorbent fibrous strip which can be dipped into said urine. 20

10. A method according to Claim 1 in which said 20- α reagent is radio-labelled and the amount of unreacted 20- α reagent is determined by radio assay.

11. A test strip for use in determining the concentration of pregnanediol glucuronide in a 25 sample of a woman's urine, comprising an adsorbent fibrous strip having immobilized thereon a known quantity of a 20- α reagent consisting essentially of 20 α -hydroxy-4-pregnen-3-one. 25

12. A test strip according to Claim 11 in which said strip is composed of a glass fiber paper.

13. A method for determining the concentration of pregnanediol glucuronide in a sample of a woman's urine substantially as hereinbefore described with reference, where appropriate, to 30 any one of the Examples 1-6. 30

14. A test strip for use in determining the concentration of pregnanediol glucuronide in a sample of a woman's urine substantially as hereinbefore described with reference, where appropriate, to any one of Examples 1-6.